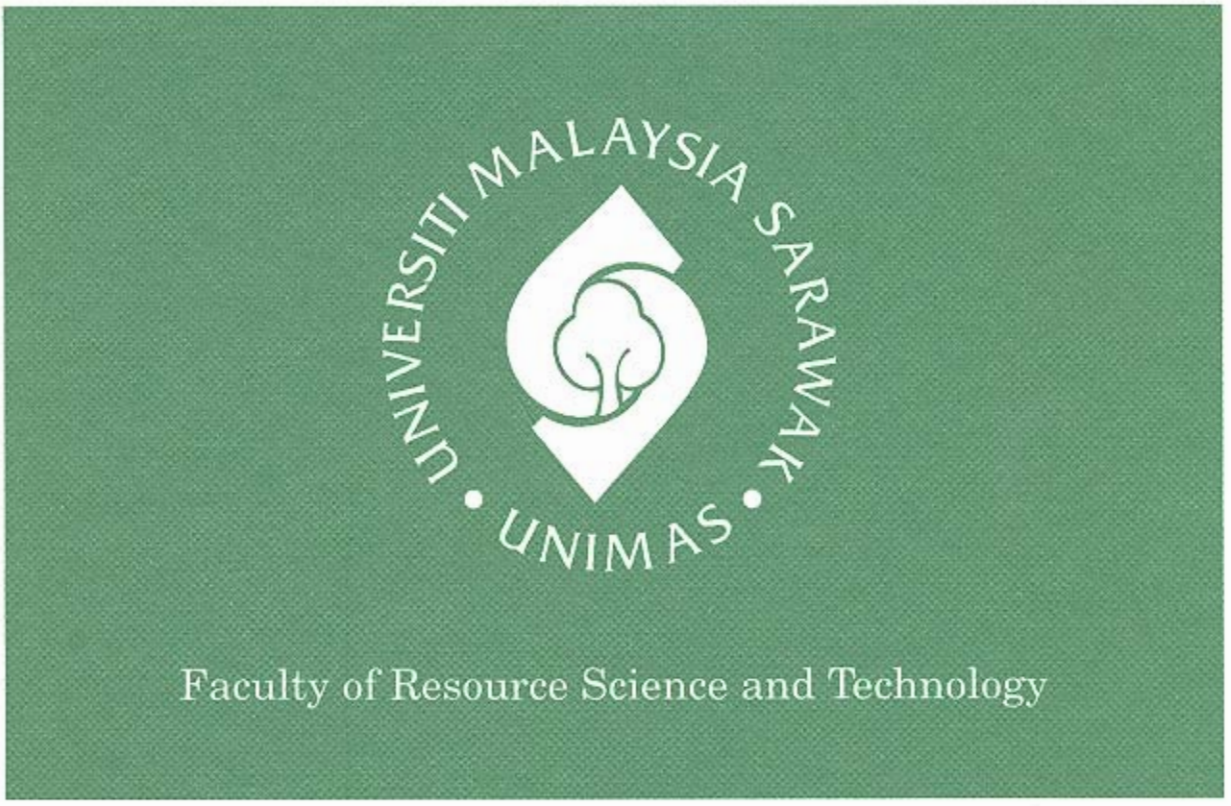

ISOLATION AND R. E SITE CHARACTERIZATION OF MITOCHONDRIAL *cytochrome b* GENE OF THE INSECTIVOROUS BAT, GENUS *Hipposideros*

Zaitul-Najahah Bt Abd. Patah

Bachelor of Science with Honours
(Resource Biotechnology)
2006

ISOLATION AND R.E SITE CHARACTERIZATION OF MITOCHONDRIAL *cytochrome b* GENE OF THE INSECTIVOROUS BAT, GENUS *Hipposideros*

ZAITUL-NAJAHAH BT ABD. PATAH

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITY MALAYSIA SARAWAK
2006

Isolation and R.E site characterization of mitochondrial *cytochrome b* gene of the insectivorous bat, genus *Hipposideros*.

Zaitul-Najahah Binti Abd Patah

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak
94300 Kota Samarahan
Sarawak Malaysia

ABSTRACT

Hipposideros species in Borneo are known to have limited information about the morphological features. In this study, a simple fast and cost effective method was used to determine the variation and differentiate between species of *Hipposideros* in Borneo by employing Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). A partial fragment of *cytochrome b* gene for *H. cervinus* (LNP 046) and *H. galeritus* (LNP 030) with a predicted size of 470bp was successfully amplified and purified. The result for PCR-RFLP showed different cleavage patterns of *H. cervinus* (LNP 046) and *H. galeritus* (LNP 030) by *Tru*9I, *Taq*I and *Bsu*RI restriction enzymes. This diagnostic digestion profiles showed variations that could discriminate between species of *Hippposideros* in Borneo.

Key words: cytochrome b gene (*cyt b*), Polmerase Chain Reaction-Restriction Fragments Length Polymorphism (PCR-RFLP), Restriction Enzyme (RE), *Hipposideros*, variation.

ABSTRAK

Kelawar dari genus Hipposideros di kawasan Borneo didapati mempunyai maklumat yang terhad tentang ciri morfologinya. Dalam kajian ini, metodologi yang mudah, cepat dan murah digunakan untuk menentukan kepelbagaian dan membezakan diantara spesis kelawar dari genus Hipposideros di kawasan Borneo dengan menggunakan analisis "Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)". Fragmen gen cytochrome b dengan saiz 470 bp telah berjaya diamplifikasikan dan ditulinkan. Keputusan dari PCR-RFLP menunjukkan bentuk pemotongan yang berlainan oleh spesis H. cervinus (LNP 046) dan H. galeritus (LNP 030) oleh enzim H. galeritus (LNP 030) by Tru9I, TaqI dan BsuRI. Profil pemotongan tersebut telah membuktikan bahawa terdapat kepelbagaian untuk menentukan perbezaan diantara spesis kelawar dari genus Hipposideros.

Kata kunci : Gen cytochrome b (cyt b), Polmerase Chain Reaction-Restriction Fragments Length Polymorphism (PCR-RFLP), Restriction Enzyme (RE), Hipposideros, kepelbagaian.

Acknowledgement

Firstly, I would like to say Alhamdulillah thank you ALLAH because with bless of ALAH, I successfully finished this study. Then, I would like to thank to Dr. Edmund Sim my supervisor for giving me the chance to conduct this study under him and for all support that he gave me during the period of this study. I also would like to say thank you to the entire postgraduate students under Dr. Edmund Sim especially to Imelda Paul who helped me a lot while doing this study by sharing her knowledge and experience. Laboratory Assistant Ms Lim Ja Tai and also to the research assistants also under Dr. Edmund Sim. Lastly, I would like to thank to my friends who is also under the same supervisor and the important persons in my life which is my parent for their support and help.

Table of Content

1.0 INTRODUCTION

Bats are the second-most speciose group of mammals, after rodents. There are two major suborder of bats, Megachiroptera and Microchiroptera. The majority of the microchiropteran species are insectivorous. Insectivorous bats or Microchiropterans, account for almost a quarter of all mammal species. They have enormous ecological benefits in the sheer volume of insects. The targeted samples that were used in this study were the genus *Hipposideros*. *Hipposideros* consist of 11 species in Borneo and are also known as the roundleaf bats. This type of bats were chosen for the study because there are still lack of information about this genus in Borneo (Payne *et al.*, 1998)

Mitochondrial DNA (mtDNA) *cytochrome b* gene (Figure 1) was used to perform a molecular phylogeny relationship of the genus *Hipposideros* in Borneo for this study. *Cytochrome b* gene that was used is the best-known mitochondrial gene with respect to its structure and function. Its sequences have been widely used in phylogenetics research (Esposti *et al.*, 1993).

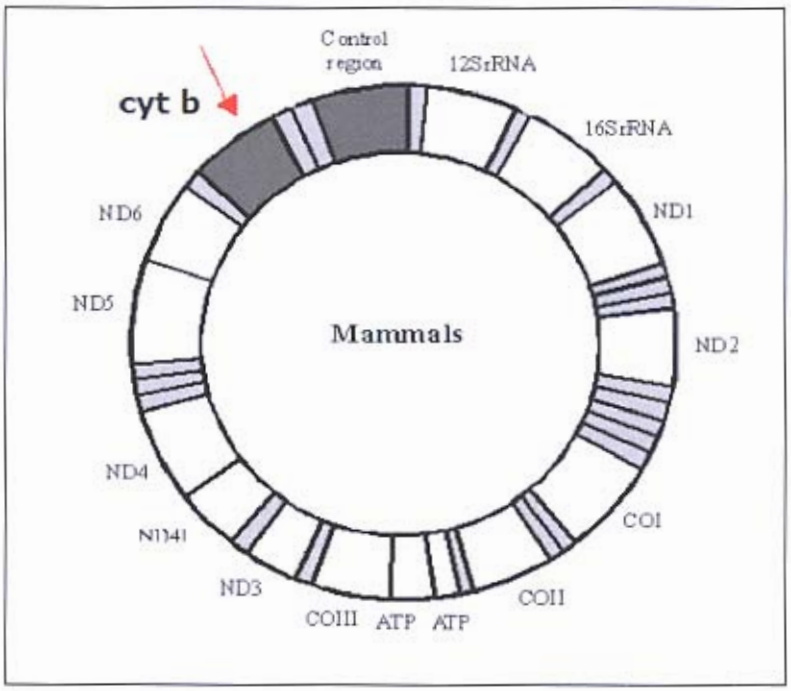

Figure 1: Mitochondrial genomes of mammals (Mindell *et al.,* 1998) adopted from www. Mitomap.org/mitomap/mitomap genome pdf. The pointer showed the *cyt b* gene site of the mammal's mtDNA.

Polymerase Chain Reaction (PCR) in this study was used to amplify the mithochondrial DNA *cytochrome b* gene that had been isolated from the genomic DNA extraction. PCR is a technique for amplifying a specific region of DNA. The DNA synthesis is initiated by a thermostable DNA polymerase. Gel electrophoresis was used to detect the PCR product.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used in this study as an alternative technique to DNA sequencing for molecular phylogenetics study.aaaaccording to Futoshi et al., (2005), PCR-RFLP is a simple, inexpensive and promising technique among the DNA-based analytical methods. This technique is generated by PCR amplification because of its easy application. This technique also requires neither expensive equipments nor costly nucleotide sequencing and is a technique that differentiated by analysis of patterns derived from cleavage of their DNA with the restriction enzymes. The length of the fragments produced was different when the DNA had been digested with a restriction enzyme (Futoshi *et al.*, 2005). Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme. Enzyme recognition sites are usually 4 to 6 base pairs in length. The similarities and differentiation of the patterns generated are used to characterized the species between *Hipposideros* in Borneo in this study.

2.0 OBJECTIVES

This study was done to determine the variation and the differentiation between the species in the genus *Hipposideros* using the mitochondrial *cytochrome b* gene via PCR-RFLP. The variation could be determined by using restriction enzymes that cleavage specific nucleotide on the restriction site of the *cytochrome b* gene.

2.1 RESEARCH QUESTION AND RATIONALE

There are limited external characteristic among morphologically similar species of *Hipposideros*. This research was done to isolate *cytochrome b* gene from genus *Hipposideros* and characterized using the restriction enzymes. This study is also to determine the variation that occurs among the species within the genus *Hipposideros* in Borneo using the mitochondrial *Cytochrome b* gene via PCR-RFLP.

3.0 LITERATURE REVIEW

3.1 Microchiropteras

According to Payne *et al.*, (1998), bats are thought to have originated between 65 and 100 million years ago from an insectivore ancestor. There are two major groups of bats, the suborders, Megachiroptera (megabats) and Microchiroptera (microbats). A majority of the species are insectivorous bats. The Microbats or also known as insectivorous bats develop the ability of using echolocation for foraging and manoeuring. These Microbats have small body size, small eyes and sharped pointed teeth. They feed on insects as a main diet.

3.2 Classification and characteristics of *Hipposideros*

According to description by Payne *et al.*, (1998), there are 73 species of microchiroptera in Borneo and 11 species in the genus *Hipposideros* or roundleaf bats; *H. diadema, H. larvatus, H. dyacorum, H. ater, H. cineraceus, H. bicolor, H. coxi, H. ridleyi, H. galeritus, H. cervinus and H. sabanus*. *Hipposideros* are the typical horshoe-shaped anterior noseleaf. The skull is generally similar to *Rhinolophus*, but rostrum with paired swellings on top. Anterior upper premolar variably reduced; upper canines heavy but simple, without prominent supplementary cusps (Payne *et al.*, 1998)

3.3 Mitochondrial DNA (mtDNA)

The analysis on mitochondrial DNA has been developed for evolutionary studies of many animal species including Chiroptera (Meyer *et al.*, 1990) with the advent of the polymerase chain reaction (PCR) and the discovery of universal PCR primers for mitochondrial DNA sequences (Kocher *et al.*, 1989). Animal mtDNA is maternally inherited (Avise, 1986).

3.4 *Cytochrome b* gene

Cytochrome b is one of the best known proteins that make up complex III of the mitochondrial oxidative phosphorylation system (Hatefi, 1985) and is the only one encoded by the mitochondrial genome. *Cytochrome b* is a transmembrane protein consisting of eight-helices and it is believed to contain both redox centers Qo and Qi (Hatefi, 1985). According to Trumpower (1990), all eukaryotic organisms require this class of redox enzymes, and consequently *cytochrome b*, for energy conservation. The mitochondrial *cytochrome b* gene is indicated to be of ancient origin (Eposti *et al.*, 1993) and shows a relatively high mutations to enable discrimination of a wide variety of fish, even closely related species belonging to the same families and genera (Chow *et al.*, 1993; Ram *et al.*, 1996; Quinteiro *et al.*, 1998; Lindstrom 1999; Russel *et al.*, 2000; Jerome *et al.*, 2003). Based on mutational studies a structural model of *cytochrome b* including the sites of electron transfer and inhibitor action has been developed (Howell and Gilbert, 1988; di Rago *et al.*, 1990). The At present *cytochrome b* gene is also used as a tool in studies of molecular evolution (Kocher *et al.*, 1989; Montgelard *et al.*, 1997; Prusak *et al.*, 2004) and legal medicine (Bartlett and Davidson, 1992; Zehner *et al.*,

1998; Parson *et al.*, 2000). *Cytochrome b* gene has been completely or partially sequenced for many species of mammals, birds, reptiles, amphibians, fishes and also some invertebrates.

3.5 Restriction Fragment Length Polymorphism (RFLP)

There are many analyses which use PCR-RFLP technique, especially in forensic laboratory. Slight but unique differences in the banding pattern of DNA fragments from different individuals of a species are observed when subjected to restriction enzyme analysis. These variations in the DNA are used as markers on both physical and genetic linkage maps. Such differences in the RFLP profiles have revolutionized criminal investigations and have become powerful tools in the identification of individuals in paternity and maternity cases, population genetics, and in the diagnosis of a variety of diseases. Restriction fragment length analyses uses restriction enzymes (RE) to cut DNA at specific 4-6 bp recognition sites (Dowling *et al.*, 1990). Sample DNA is cut (digested) with one or more retriction enzymes and resulting fragments are separated according to molecular size using gel electrophoresis (Avise 1994). Molecular size standards are used to estimate fragment size. Ethidium bromide staining is used to reveal the fragments under UV (260 nm) light. RFLP is most suited to studies at the intraspecific level or among closely related taxa. Presence and absence of fragments resulting from changes in recognition sites are used identifying species or populations.

4.0 MATERIAL AND METHODS

4.1 Material

4.1.1 Sample collection

The source were taken from old samples of preserved blood and tissue samples of *Hipposideros* bats that had been used in previous studies were taken from UNIMAS museum collection From 11 species, only 9 species were found as UNIMAS museum collection. Another source that had been used in this study was the fresh samples collected from the field trip in Lambir National Park, Miri, Sarawak from 30th January 2006 to 4th February, 2006. Only six species were collected in Lambir National Park compared to the number of species that were used before and taken from UNIMAS museum.

4.2 Methods

4.2.1 DNA Extraction

Isolation of genomic DNA from blood and muscle tissues samples was constructed using commercial kits (Viogene® Blood and Tissue Genomic extraction System). The protocol that was used is as follows: The sample tissues were washed with PBS buffer and were minced into fine pieces and were then placed in a sterile 1.5 ml Eppendorf tube. Then, 200µl of lysis buffer was added, followed by 20 µl Proteinase K. The mixture was left for overnight incubation at 60°C. For the next step, the incubator

need to be adjusted to 70°C and then the mixture that had been incubated overnight were incubated for 20 minutes. Then, 200 µl of EX buffer was added to the sample and was mixed by vortexing. The sample that had been added was then incubated again at 70°C for 10 minutes. After the incubation, 210 µl of absolute ethanol was added and mixed by vortexing. The mixture was then transferred to the B/T Genomic DNA Mini Column was placed onto a collection tube and was centrifuged at 8000 rpm for two minutes and the column placed onto a new collection tube. Then, 0.5ml of WS buffer was used twice to wash the filtrate by centrifuging at 8000 rpm for two minutes. The flow through was discarded. The column was then centrifuged at full speed for another two minutes to remove the ethanol. Then the column was placed onto a new sterile 1.5 ml microcentrifuge tube. After that, 200 µl of preheated sterile distilled water was used to elute DNA. The column was left to stand for one minute before it was centrifuged for two minutes at full speed. The eluted DNA was stored at -20°C. The protocol of the blood extraction is same with tissue samples but for the blood sample, it does not need the incubation overnight step but it was incubated in 60°C for 20 minutes after mixed with the Proteinase K. The mixture was inverted at every three to five minute intervals.

4.2.2 Analysis of genomic DNA by Spectrophotometer

In spectrophotometer method, 5µl of sample DNA was pipetted into the cuvette and 495µl of sterile water was added to make up to 500µl. The cuvette was then placed in spectrophotometer and the DNA concentration was then analyzed. The calculation for the concentration is: Concentration = Absorbance x Extinction coefficient x dilution factor (DF) x path length.

4.2.3 PCR Amplification

Polymerase chain reaction (PCR) was performed to amplify the *cytochrome b* gene. Two types of primers were used to amplify the *cyt b* gene (Table 1).

Table 1: Sets of primer used in PCR amplification of cytochrome b gene and predicted size of each *cyt b*

Sets of Primer	Type	Primer name	Primer sequence	Expected size of product
1.	Forward	L14724	5' – CGA AGC TTG ATA TGA AAA ACC ATC GTT G – 3'	1400bp
	Reverse	H15915	5' – AAC TGC AGT CAT CTC CGG TTT ACA AGA C – 3'	
2.	Forward	Glud G-L	5' – TGA CCT GAA RAA CCA YCG TTG – 3'	450bp
	Reverse	CB2H	5' – CCT TCA GAC TGA TAT TTG TCC TCA – 3'	

Each of the PCR amplification using respective sets of primers was performed using a total of 25µl PCR reaction mixture. The exact amount of each ingredient in each PCR reaction mixture (Table 2):

Table 2: Ingredient and their appropriate amount used in each PCR reaction

Ingredient	Amount (µl)
5 X Green buffer (Promega, USA)	10
25mM $MgCl_2$	2
10mM dNTP mix	1
25 pmol/µl Forward Primer	2.5
25 pmol/µl Reverse Primer	2.5
5 U/µl *GoTaq* polymerase (Promega, USA)	0.25
Sterile ultrapure water	1.75
Template DNA	5

Each PCR process consisting of 35 cycles was carried out using thermal cycler (Perkin Elmer Model No. 2400) using the following parameters (Table 3):

Table 3: PCR parameters

Stages	Temperature (°C)	Duration
Denaturation	94.0	40 seconds
Annealing	Varies according to the individuals	2 minutes
Extension	72.0	1 minute
Final extension	72.0	10 minutes

Before starting the PCR process, all samples were incubating in 95.0°C for about 10 minutes. All the PCR mixture that were stated in Table 3 was mixed together except the *GoTaq* polymerase that was insert only after the incubation.

4.2.4 Gradient Thermal PCR

This method was used to determine the optimal annealing temperature for the *cytochrome b* (complete) region primer. According to Kocher *et al.*, (1989), there were two *cytochrome b* primers for complete region were used;

L14724 5'– CGA AGC TTG ATA TGA AAA ACC ATC GTT G– 3' as forward primer

H15915 5' – AAC TGC AGT CAT CTC CGG TTT ACA AGA C – 3' as reverse primer

The Gradient PCR amplification was carried out in 12.5µl PCR reaction mixture. The PCR reaction was showed in table 4.

Table 4: Ingredient and their appropriate amount used in each Gradient PCR reaction

Ingredient	Amount (µl)
10 X Green buffer (Promega, USA)	1.25
25mM $MgCl_2$	0.5
10mM dNTP mix	0.25
10 pmol/µl Forward Primer	1.25
10 pmol/µl Reverse Primer	1.25
5 U/µl *GoTaq* polymerase (Promega, USA)	0.05
Sterile ultrapure water	6.95
Template DNA	1

Each PCR process consisting of 35 cycles was carried out using thermal cycler (Biometra). The parameter for gradient PCR in this study was different. This was discussed in the results section.

4.2.5 Analysis of genomic DNA and PCR product and PCR-RFLP by Gel Electrophoresis

All samples were screened using the gel electrophoresis in 1.5% agarose (0.6g of agarose powder and 40ml of TAE buffer) gel in 50ml of 1X TAE buffer at 95 volt for 30 minutes to determine the genomic DNA and also for PCR product. The product for RFLP was performed by using the 3.0% agarose (1.2g of agarose powder and 40 ml of TBE buffer) gel in 50 ml of 1X TBE buffer at 40 volt for 2 hours. For each DNA samples and gradient PCR products, 10µl was mixed with 2 µl of 6X gel loading dye and subsequently loaded into wells. For each PCR product, 10 µl of the product were loaded into the wells without using the loading dye because it already had the green buffer that can be as the loading dye. Then, 3µl of GeneRuler® 1 kb DNA ladder (Fermentas, USA) for DNA samples, Gradient PCR and PCR product and 3µl of

GeneRuler® 50bp DNa ladder (Fermentas, USA) for PCR-RFLP product was loaded into an adjacent well as a standard size marker for DNA fragments. The gel was stained in ethidium bromide at 0.5 μg/ml for 30 minutes. Then, the gel was then viewed under a UV transluminator and images were captured onto 667 black and white Polaroid films using Ultralum's Direct Screen Instant Polaroid Camera DS34.

4.2.6 Gel Extraction

To proceed to PCR-RFLP method, the product of PCR product must be extract. The extraction was done using the Viogene® Gel Extraction System according to manufacturer's instruction. The protocol that was used was as the following: DNA fragment of interest from the gel that was viewed under the UV light was excised using a clean and sharp scalpel. Then the gel slice was transferred into 1.5 microcentrifuge tube. 0.5 ml of GEX buffer was added into the tube containing the gel slice and incubated in 60°C for 10 minutes to solubilize the gel slices. Then the solubilized gel mixture was transferred into a gel extraction column in a collection tube and centrifuged at 13000 rpm for 60 second. The filtrate was discarded and the column was further washed with 500μl of WF buffer and centrifuged at 13000 rpm for 60 second. The flow through was discarded and the filtrate was washed with 700μl WS buffer which contain the ethanol and again was centrifuge at 13000 rpm for 60 second. Following washing, remaining ethanol residue was removed by further centrifugation at full speed (14000) for 3 minutes. The column was then place to a new microcentrifuge tube. 35μl Elution Buffer was added onto the centre of the membrane of the column. Centrifugation was

carried out at 14000 rpm for 2 minutes after the column was left to stand for 1 minute. 5µl of the filtrate was screened for purified DNA and the remaining filtrate was stored at -20°C.

4.2.7 PCR-RFLP

The site of restriction endonuclease and the types of the restriction enzymes that were used in this study was determined by using the WebCutter 2.0 software system. The FASTA format of sequence of the partial *cyt b* gene was taken from the National Centre of Biotechnology Information using the accession number AF451334 for *H. bicolor* and was copied and paste to the WebCutter 2.0 software system to determine the restriction endonulease site and types of restriction enzymes that could be used. The types of enzymes that were provided and same with the type of restriction enzymes that listed by the software were used. The protocol of PCR-RFLP is as follows: 4µl of the sterile distilled water was added to the microcentrifuge tube. Then, 1µl of the restriction enzyme buffer was added to the sterile distilled water. Then the 4µl purified DNA which contain the *cyt b* gene was then added to the mixed sterile and restriction enzyme buffer. Lastly, 1µl of the restriction enzymes was then added to the mixture. Total volume for this PCR-RFLP method is 10µl. The mixture was then incubated overnight according to the optimum temperature of each restriction enzymes that were used.

5.0 RESULTS

5.1 DNA extraction

Preserved blood and tissue samples of *Hipposideros* that had been extracted were analyzed by using the gel electrophoresis to screening the genomic DNA. The diagram of the genomic DNA that was visualized by the gel documentation system under UV transluminator was showed in figure 2. Based on the results in figure 2 we can only observed that samples in lane number 14 (H. *larvatus*) showed evidenced of genomic DNA. Others samples showed smears and the rest were negative results.

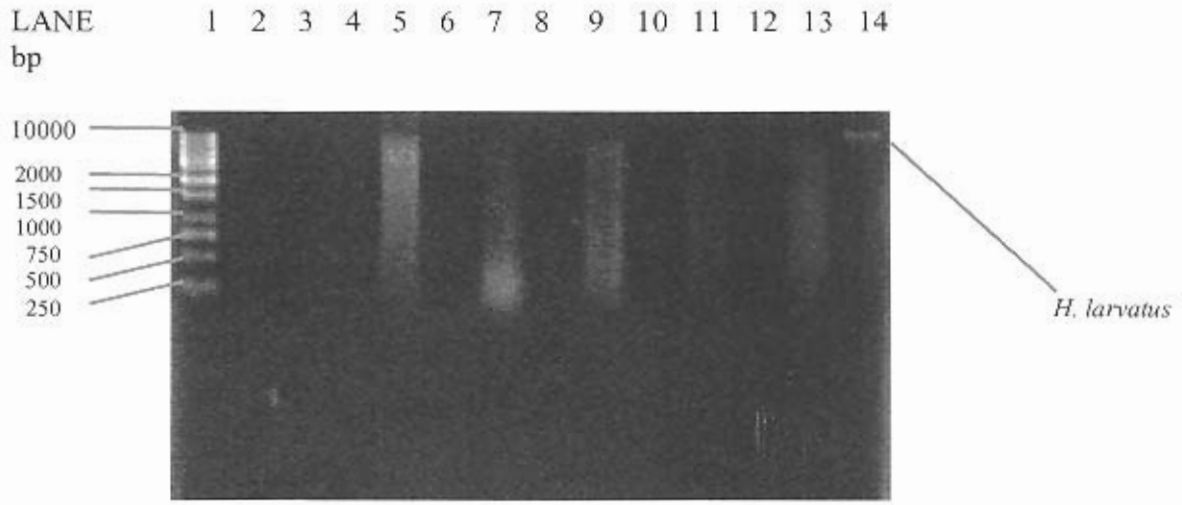

Figure 2: 1.5% Agarose gel electrophoresis of total genomic DNA of *Hipposideros*. Lane 1 represents the GeneRuler® 1 kb DNA ladder. Lane 2-14 represents *H. bicolor* (B125), *H. bicolor* (124), *H. galeritus* (B165), *H. ridleyi* (B160), *H .cervinus* (B126), *H. dyacorum* (B096), *H. larvatus* (B120), *H. larvatus* (B148), *H. cervinus* (B166), *H. cervinus* (B166), *H. bicolor* (B124), *H .coxi* (B145) and *H. larvatus* (B148).

After the results on the gel had been determined, all samples were analyzed using the spectrophotometer to determine the purity ($A_{260/280}$ nm) and the concentration of DNA ($A_{260/230}$ μg/μl) of the samples. The analysis results of the spectrophotometer analysis results (Table 5):

Table 5: Spectrophotometer analysis results for DNA samples of *Hipposideros*

Sample number	Species	Purity of DNA	DNA Concentration (μg/μl)
B120	*H. larvatus*	2.410	0.054
B120	*H. larvatus*	2.304	0.035
B124	*H. bicolor*	2.181	0.012
B124	*H. bicolor*	2.517	0.060
B125	*H. bicolor*	26.913	0.009
B126	*H. cervinus*	1.492	0.069
B148	*H. larvatus*	1.940	0.381
B166	*H. cervinus*	1.629	0.237
B166	*H .cervinus*	1.683	0.020
B165	*H. galeritus*	1.624	0.026
B160	*H. ridleyi*	1.792	0.026
B145	*H. coxi*	1.907	0.096
B096	*H. dyacorum*	1.805	0.160
B126	*H. cervinus*	2.049	0.068
B119	*H. ater*	1.716	0.031

5.2 PCR Optimization and Amplification

The results for the DNA extraction of the fresh samples that were collected from a field trip in Lambir National Park, Miri Sarawak were showed in figure 3 and figure 4. Five samples of *Hipposideros* that used in this study which were existed in this area which are *H. cervinus* (LNP 001), *H.galeritus* (LNP 030), *H. cervinus* (LNP 046), *H. diadema* (LNP 123), and *H. ridleyi* (LNP 109) produced a clear single linear band of DNA.

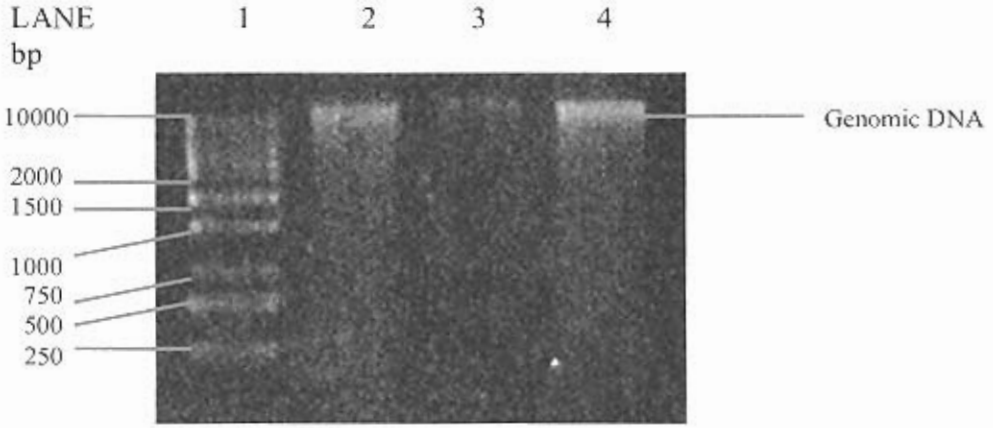

Figure 3: 1.5% Agarose gel electrophoresis of total genomic DNA of *Hipposideros* of fresh samples that were collected from Lambir National Park. Lane 1 represents the GeneRuler® 1 kb DNA ladder. Lane 2-3 represents for *H. ridleyi* (LNP 109), *H. diadema* (LNP 123), and *H. cervinus* (LNP 046).

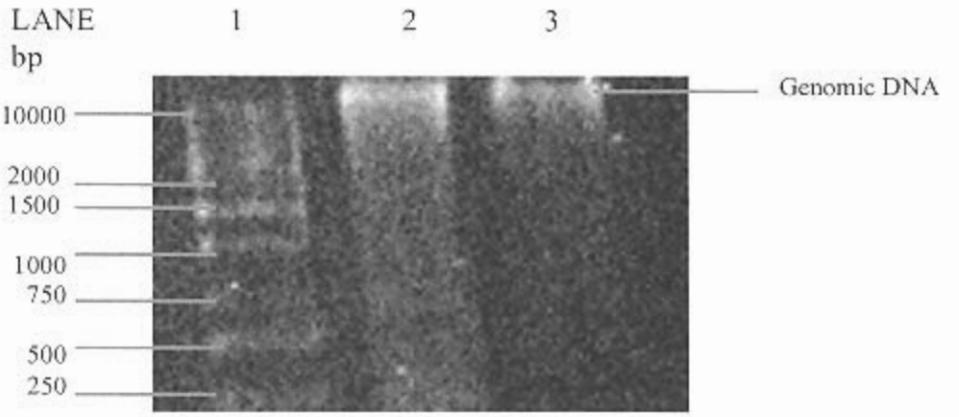

Figure 4: 1.5% Agarose gel electrophoresis of total genomic DNA of *Hipposideros* of fresh samples that were collected from Lambir National Park. Lane 1 represents the GeneRuler® 1 kb DNA ladder. Lane 2-3 represents for *H. galeritus* (LNP 030) and *H. cervinus* (LNP 001).

Optimization of the annealing temperature by using the gradient thermal PCR for complete *cytochrome b* primer was done after analyzing the genomic DNA using the gel electrophoresis and the spectrophotometer. The parameters that had been tested in this study were as in table 6 to table 9. From the entire test, only the results for the parameter in table 8 and table 9 were showed in figure 5 and figure 6. Overall of the results, the complete *cytochrome b* could not be optimized because the results for all parameters showed only primer dimmers and smears.